Role of Kaposi’s Sarcoma-Associated Herpesvirus C-Terminal LANA Chromosome Binding in Episome Persistence†‡

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Kaposi’s sarcoma-associated herpesvirus (KSHV) LANA is an 1,162-amino-acid protein that tethers terminal repeat (TR) DNA to mitotic chromosomes to mediate episome persistence in dividing cells. C-terminal LANA self-associates to bind TR DNA. LANA contains independent N- and C-terminal chromosome binding regions. N-terminal LANA binds histones H2A/H2B to attach to chromosomes, and this binding is essential for episome persistence. We now investigate the role of C-terminal chromosome binding in LANA function. Alanine substitutions for LANA residues 1068LKK1070 and 1125SHP1127 severely impaired chromosome binding but did not reduce the other C-terminal LANA functions of self-association or DNA binding. The 1068LKK1070 and 1125SHP1127 substitutions did not reduce LANA’s inhibition of RB1-induced growth arrest, transactivation of the CDK2 promoter, or C-terminal LANA’s inhibition of p53 activation of the BAX promoter. When N-terminal LANA was wild type, the 1068LKK1070 and 1125SHP1127 substitutions also did not reduce LANA chromosome association or episome persistence. However, when N-terminal LANA binding to chromosomes was modestly diminished, the substitutions in 1068LKK1070 and 1125SHP1127 dramatically reduced both LANA chromosome association and episome persistence. These data suggest a model in which N- and C-terminal LANA cooperatively associates with chromosomes to mediate full-length LANA chromosome binding and viral persistence.

Kaposi’s sarcoma (KS)-associated herpesvirus (KSHV), or human herpesvirus 8, is a gamma-2 herpesvirus. Clinically, it is associated with KS, multicentric Castleman’s disease, and primary effusion lymphoma (10, 16, 17, 55, 73). KSHV infection is predominantly latent, with multiple copies of the viral genome persisting in the nuclei of dividing cells (11, 14). Latency-associated nuclear antigen (LANA) is among a small subset of viral genes expressed during latency. LANA is a multifunctional protein that regulates both transcription and cell growth. LANA also affects the KSHV latency program of gene expression (42, 43).

LANA regulates transcription from a variety of cellular and viral promoters, repressing some genes while activating others (1, 15, 26, 38, 41, 46, 50, 57, 59, 67, 69, 76, 77, 85). For example, LANA represses p53-driven transcription of the proapoptotic protein BAX (79), but LANA activates transcription of E2F1 (1, 15, 26, 38, 41, 46, 50, 57, 59, 67, 69, 76, 77, 85). LANA associates with mitotic chromosomes (2, 40, 79) to mediate episome replication and persistence (3, 13, 20, 25, 26, 31, 32, 39, 47, 83). LANA also induces B-cell hyperplasia and lymphomas in transgenic mice, and LANA prolongs the life span of primary endothelial cells (19, 78). In addition, LANA modulates the Wnt signaling pathway by binding to and altering the activity of glycogen synthase kinase-3β, resulting in increased levels of β-catenin (22–24, 48). β-Catenin accumulation is a common characteristic of primary effusion lymphoma and KS cells, suggesting the importance of its deregulation in the development of these cancers (24). LANA also stabilizes c-Myc, which is frequently dysregulated in cancer (9, 49).

In addition to regulating gene transcription and affecting cell growth pathways, LANA mediates episome persistence by tethering KSHV terminal repeat (TR) DNA to host cell chromosomes. C-terminal LANA binds cooperatively to two sites within each TR element; this binding is necessary for both episome replication and persistence (3, 13, 20, 25, 26, 31, 32, 39, 47, 83). LANA associates with mitotic chromosomes (2, 40, 61, 75), and N- and C-terminal LANA contains independent chromosome binding regions (4, 36, 37, 40, 45, 61, 79). N-terminal LANA binds to core histones H2A/H2B to attach to chromosomes, and this binding is necessary for LANA-mediated KSHV DNA replication and episome persistence (4, 5).

This study investigates C-terminal LANA chromosome binding. Previous work identified mutants with severely im-

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paired C-terminal LANA chromosome attachment (37). We find that when N-terminal LANA binding to chromosomes is modestly diminished, impaired C-terminal LANA chromosome binding greatly reduces both LANA chromosome association and episome persistence. Based on this evidence, we propose a model in which N- and C-terminal LANA binds cooperatively to mitotic chromosomes to mediate efficient persistence of TR DNA.

MATERIALS AND METHODS

Cell lines. KSHV-infected BCBL-1 cells were maintained in RPMI medium containing 20% bovine growth serum (BGS) (HyClone). BJAB cells were maintained in RPMI medium containing 10% BGS or FetalClone (Gemini). H1299 and Saos2 cells were maintained in Dulbecco's modified Eagle's medium containing 10% and 25% BGS, respectively.

Plasmids. GFP NLS contains a nuclear localization signal (NLS) cloned in-frame downstream of green fluorescent protein (GFP) in EGF-C1 (Clontech) (34). GFP LANA residues 933 to 1162 (GFP LANA 933–1162) contains the indicated LANA residues downstream of the NLS in GFP NLS. GFP LANA 933–1162 1125SHP1127 have the indicated residues mutated to alanines (37). A fragment of GFP LANA 933–1162 1125SHP1127 containing GFP LANA residues 1075 to 1162 was subcloned into GFP LANA 933–1162 1068LKK1070 to generate GFP LANA 933–1162 1068LKK1070/1125SHP1127. C-terminal LANA alanine substitution mutations were subcloned into GFP LANA 933–1162 1068LKK1070/1125SHP1127. A segment of GFP LANA 933–1162 1077FGG1079 was amplified and inserted into GFP LANA 933–1162 1068LKK1070 to generate GFP LANA 933–1162 1068LKK1070/1077FGG1079. GFP LANA 933–1162 1125SHP1127 alanine substitutions might completely abolish C-terminal LANA chromosome association without disrupting DNA binding. In KSHV 933–1162 1068LKK1070 1125SHP1127 severely compromise C-terminal LANA chromosome binding (Fig. 1A) without impairing LANA self-association or DNA binding (37). However, neither the 1068LKK1070 nor 1125SHP1127 mutation results in complete loss of C-terminal LANA mitotic chromosome attachment.

We considered the possibility of a model in which C-terminal alanine substitutions might completely abolish C-terminal LANA chromosome association without disrupting DNA binding. We generated the double mutant GFP LANA 933–1162 1068LKK1070 1125SHP1127 severely compromise C-terminal LANA chromosome binding (Fig. 1A) without impairing LANA self-association or DNA binding (37). However, neither the 1068LKK1070 nor 1125SHP1127 mutation results in complete loss of C-terminal LANA mitotic chromosome attachment.

We wondered whether combining 1068LKK1070 and 1125SHP1127 alanine substitutions might completely abolish C-terminal LANA chromosome association without disrupting DNA binding. We generated the double mutant GFP LANA 933–1162 1068LKK1070 1125SHP1127. GFP LANA 933–1162 1068LKK1070 was not transfected using an Opto-comp-1 luminometer (MGM Instruments) and luminescent reagents (Promega and Applied Biosystems). Luciferase activity was normalized to that of ß-galactosidase and expressed as the relative increase above reporter alone. Values are the averages of three independent transfections, each assayed in triplicate, with error bars indicating the standard deviation from the mean.

RESULTS

Simultaneous mutation of residues 1068LKK1070 and 1125SHP1127 does not further impair C-terminal LANA chromosome association. C-terminal LANA self-associates to bind TR DNA, and this binding is essential for LANA-mediated DNA replication and episome persistence (39). Therefore, in order to investigate the episome maintenance function of C-terminal LANA chromosome association, it is critical to disrupt only chromosome association and not other essential C-terminal LANA functions. C-terminal LANA deletions of ~15 amino acids result in complete loss of C-terminal LANA chromosome association, but these deletions also abolish LANA self-association and TR DNA binding (37, 39). Alanine substitutions for 1068LKK1070 or 1125SHP1127 severely compromise C-terminal LANA chromosome binding (Fig. 1A) without impairing LANA self-association or DNA binding (37). However, neither the 1068LKK1070 nor 1125SHP1127 mutation results in complete loss of C-terminal LANA mitotic chromosome attachment.

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Flat-cell assay. Using Lipofectamine 2000 (Invitrogen), Saos2 cells (~4 × 10^6) were transfected with 1 µg of a plasmid conferring puromycin resistance (56), 10 µg of pCMV-Rb, and 20 µg of pSG5 F-LANA or F-LANA mutant. At 24 h posttransfection, cells were replated at low density (0.5 × 10^5 cells per 1-cm dish). Selection with 0.3 to 0.5 µg/ml puromycin (Sigma) was initiated 24 h after replating and continued for 8 to 10 days, at which time all flat cells within the central 12.6-cm^2 circular region of each dish were counted, and images were captured using the Zeiss Axiosvert 200 and Axiovision software.

Selection of G418-resistant cells and Gardella gel analysis. BJAB cells stably expressing F-LANA or F-LANA mutants were cotransfected with GFP and p8TR. Alternatively, BJAB cells were cotransfected with GFP and pLANA 14TG15-8TR, pLANA 14TG15/1068LKK1070-8TR, pLANA 14TG15/1077FGG1079-8TR, pLANA 14TG15/1125SHP1127-8TR, or pZ6e8TR. At 48 to 72 h posttransfection, LANA or LANA mutant expression was assayed by immunoblotting. Cells were seeded in 96-well microtiter plates at various concentrations (1, 10, 100, or 1,000 cells/well) in medium containing G418 (60 U/ml) (Gibco). Cell growth in microtiter plates was recorded 15 to 19 days after seeding. Gardella analysis was performed by in situ lysis of cells in gel-loading wells with pronase and sodium dodecyl sulfate (27). Following electrophoresis in Tris-borate-EDTA, DNA was transferred to a nylon membrane, and KSHV DNA was detected using a 32P-labeled TR probe.
dots on a subset of mitotic chromosomes, consistent with prior results (36, 37). As expected, GFP LANA 933–1162 1068LKK1070 and GFP LANA 933–1162 1125SHP1127 were severely compromised for mitotic chromosome association. These mutants were distributed both diffusely and in dots, and the diffuse distribution was both on and off of chromosomes (Fig. 1B) (37). In contrast, GFP NLS diffusely distributed throughout the cell but did not associate with chromosomes (Fig. 1B). GFP LANA 933–11621068LKK1070/1125SHP1127 exhibited both diffuse and dotted distribution both on and off of mitotic chromosomes, similar to the 1068LKK1070 or 1125SHP1127 mutations alone (Fig. 1B), and did not distribute only to the interchromosomal space, as did GFP NLS. Therefore, GFP LANA 933–1162 1068LKK1070/1125SHP1127 was not further impaired for chromosome binding compared with mutation of 1068LKK1070 or 1125SHP1127 alone.

We next investigated whether the 1068LKK1070/1125SHP1127 mutations affect LANA’s ability to bind TR DNA. The C-terminal LANA alanine substitution mutations were subcloned into full-length F-LANA and electrophoretic mobility shift assay performed using a TR probe containing the LANA binding site. Both wild-type F-LANA and F-LANA 1068LKK1070/1125SHP1127 complexed with TR probe with similar efficiencies (data not shown). These results were similar to those previously observed for either F-LANA 1068LKK1070 or F-LANA 1125SHP1127 (37). In conclusion, alanine substitutions for LANA residues 1068LKK1070 and 1125SHP1127 alone or in combination severely impaired, but did not completely ablate, C-terminal LANA chromosome attachment. Further, mutation of these residues had no effect on LANA binding to TR DNA. C-terminal LANA mutants with severely impaired chromosome binding have low mitotic expression indices. We previously observed that GFP C-terminal LANA mutants that are compromised for chromosome binding were detected in mitotic cells less frequently than GFP LANA 933–1162 or C-terminal LANA mutants that associated with chromosomes in a wild-type pattern (37). Further, the mutants deficient in chromosome binding were usually expressed at high levels when detected in mitotic cells. The infrequent detection was not due to unstable protein or poor expression since the mutants deficient in chromosome binding and those wild type for chromosome binding were expressed at similar levels, as assessed by

FIG. 1. LANA residues 1068LKK1070 and 1125SHP1127 are critical for C-terminal LANA chromosome association. (A) Schematic of LANA and alanine substitution mutations. Nuclear localization signals (+) (61), N-terminal chromosome binding region (shaded), central acidic repeat region, putative leucine zipper (LZ), and TR DNA and C-terminal chromosome binding domain (hatched) are indicated. The chromosome binding ability of GFP LANA 933–1162 (933–1162) or F-LANA (contains LANA residues 5 to 1162) containing each indicated mutation is summarized. Dagger denotes data from Kelley-Clarke et al. (37), with the exception of GFP LANA 933–1162 1068LKK1070/1125SHP1127. +++++, wild-type pattern; +/-, severely impaired; ND, not determined. (B) BJAB cells transiently expressing GFP NLS or GFP LANA 933–1162 mutants. Cells were arrested in mitosis and DNA counterstained with propidium iodide, and slides were analyzed by confocal microscopy. Overlay of GFP (green) and chromosomes (red) generates yellow. Magnification, ×630. (C) C-terminal LANA mutants with severely impaired chromosome attachment have low mitotic expression indices compared to wild-type C-terminal LANA. BJAB cells transiently expressing GFP NLS, GFP LANA 933–1162, or GFP LANA 933–1162 mutants were arrested in metaphase, and slides were analyzed by confocal microscopy. At least 1,500 cells in random fields were observed for each GFP fusion protein. The mitotic expression index of each mutant or GFP was calculated relative to that of GFP LANA 933–1162, as described in Materials and Methods. 933–1162, GFP LANA 933–1162; GFP, GFP NLS; LKK, GFP LANA 933–1162 1068LKK1070; SHP, GFP LANA 933–1162 1125SHP1127; and LKK/SHP, GFP LANA 933–1162 1068LKK1070/1125SHP1127.
Western blotting, and expression levels in interphase cells was similar, as observed by microscopy. A likely possibility was that the GFP C-terminal LANA mutants were lost from mitotic cells during processing of cells for microscopy, as suggested previously (37). We used a mitotic expression index to quantify the mitotic cell expression of C-terminal LANA mutants. The mitotic expression index was defined as the fraction of mitotic cells expressing each GFP fusion protein relative to the fraction of neighboring interphase cells expressing the GFP fusion protein. The mitotic expression index of mutants deficient in chromosome binding could then be directly compared to that of wild-type C-terminal LANA.

To determine the mitotic expression indices, BJAB cells were transfected with GFP NLS, GFP LANA 933–1162, GFP LANA 933–1162 1068LKK1070, GFP LANA 933–1162 1125SHP1127, or GFP LANA 933–1162 1068LKK1070/1125SHP1127. Cells were arrested in metaphase and observed by confocal microscopy, at which time interphase and mitotic cells were counted in random fields, and the mitotic expression index was calculated. GFP NLS, which does not associate with chromosomes, had a mitotic expression index that was only 6.8% of that of GFP LANA 933–1162 (Fig. 1C). The mitotic expression index of GFP LANA 933–1162 1068LKK1070 was 0.01% relative to that of GFP LANA 933–1162, and GFP LANA 933–1162 1125SHP1127 and GFP LANA 933–1162 1068LKK1070/1125SHP1127 had mitotic expression indices of 6.1% and 13.8%, respectively. These data indicate that, similar to GFP NLS, GFP LANA 933–1162 1068LKK1070 and GFP LANA 933–1162 1125SHP1127 are detected in mitotic cells over 10-fold less frequently than GFP LANA 933–1162. The double mutation of 1068LKK1070 and 1125SHP1127 together in GFP LANA 933–1162 1068LKK1070/1125SHP1127 did not further reduce the detection in mitotic cells compared with C-terminal LANA encoding either of these mutations alone. We therefore did not pursue the 1068LKK1070/1125SHP1127 double mutant any further.

Mutation of residues critical for C-terminal LANA chromosome binding does not affect mitotic chromosome localization of stably expressed, full-length LANA. Previously, we have demonstrated that amino acid deletions that abrogate C-terminal LANA chromosome binding have no effect on chromosome targeting of full-length LANA (36). However, these assays were performed in transiently transfected cells, in which overexpression of LANA may have masked subtle differences in LANA distribution along sister chromatids. Thus, we assayed whether the 1068LKK1070 or 1125SHP1127 mutations had an effect on chromosome binding of stably expressed, full-length LANA. BJAB cells stably expressing equal amounts of F-LANA, F-LANA 1068LKK1070, F-LANA 1077FGG1079, or F-LANA 1125SHP1127 were arrested in metaphase and analyzed by confocal microscopy. Alanine substitutions for residues 1068LKK1070 and 1125SHP1127 do not reduce either C-terminal LANA chromosome association or TR DNA binding (Fig. 1) (37); thus, F-LANA 1077FGG1079 was used throughout the rest of our studies as a positive control mutant.

As expected, F-LANA (Fig. 2) associated with mitotic chromosomes. While F-LANA broadly distributed across all chromosomes, the distribution pattern along chromatids was uneven, with staining occurring more intensely on some parts of chromosomes than on others. In particular, pericentric and peritelomeric chromosome regions had more intense staining. This staining pattern was reminiscent of the pericentric and peritelomeric distribution of the C-terminal LANA chromosome binding region alone (Fig. 1B) (36), suggesting that C-terminal LANA may have a role in producing this pattern. However, F-LANA 1068LKK1070, F-LANA 1077FGG1079, and F-LANA 1125SHP1127 each associated with chromosomes in a pattern similar to that of wild-type F-LANA. Thus, alanine substitutions for 1068LKK1070 or 1125SHP1127, which greatly diminish C-terminal LANA chromosome binding, did not alter the distinctive chromosome association pattern of stably expressed, full-length LANA in this assay.

LANA residues 1068LKK1070 and 1125SHP1127 are dispensable for regulating BAX and CDK2 promoter activity. We investigated whether C-terminal LANA chromosome binding has a role in LANA-mediated transcriptional regulation. The C-terminal domain of LANA suppresses p53-mediated transcription of the proapoptotic BAX gene (79). To investigate the role of LANA chromosome association in the regulation of BAX transcription, C-terminal LANA mutants severely impaired for chromosome binding were assayed for the ability to repress p53-mediated BAX transcription. The BAX promoter upstream of luciferase (pBAX-Luc) (28) was transfected into p53-null H1299 cells in combination with pHA-p53 (18) and GFP, GFP LANA 933–1162, GFP LANA 933–1162 1068LKK1070, GFP LANA 933–1162 1077FGG1079, or GFP LANA 933–1162 1125SHP1127.
p53 transactivated the BAX promoter ~75-fold over reporter alone (Fig. 3A). Cotransfection of GFP LANA 933–1162 strongly repressed the ability of p53 to activate BAX. Each of the C-terminal LANA alanine substitution mutants also repressed p53 activation of pBAX-Luc to a degree similar to that of GFP LANA 933–1162. Therefore, mutations that greatly reduce C-terminal LANA chromosome binding did not reduce the inhibition of p53 activation of BAX.

We next investigated the role of C-terminal LANA chromosome association in activating the cellular CDK2 promoter. LANA transactivation of the CDK2 promoter is reduced in the absence of N-terminal LANA chromosome binding (79). To investigate whether amino acids critical for C-terminal LANA chromosome binding also have a role in CDK2 activation, BJAB cells were cotransfected with the CDK2 promoter (pCDK2-Luc) (82) and F-LANA, F-LANA 1068LKK1070, F-LANA 1077FGG1079, or F-LANA 1125SHP1127. F-LANA transactivated the CDK2 promoter (Fig. 3B) approximately four- to sevenfold over reporter alone although some variability was observed for the activation of F-LANA 1068LKK1070 (Fig. 3B). These data indicate that reduction in C-terminal chromosome association does not reduce LANA’s transactivation of the CDK2 promoter.

1068LKK1070 and 1125SHP1127 are not required for LANA to overcome RB1-induced flat-cell phenotype. We investigated the role of C-terminal LANA chromosome binding in LANA inhibition of RB1. RB1 expression in the RB1-negative Saos2 human osteosarcoma cell line induces growth arrest, which is accompanied by a change in morphology toward an enlarged, “flat-cell” phenotype (Fig. 4A) (33). When coexpressed with RB1 in Saos2 cells, LANA inhibits this growth arrest, and therefore fewer cells exhibit the flat-cell phenotype (63).

To determine whether C-terminal LANA chromosome association has a role in LANA-mediated suppression of the flat-cell phenotype, Saos2 cells were cotransfected with a puromycin-resistance plasmid and pCMV-Rb in combination with empty expression vector, F-LANA, F-LANA 1068LKK1070 (LKK), F-LANA 1077FGG1079 (FGG), or F-LANA 1125SHP1127 (SHP). The number of flat cells was counted after 8 to 10 days of puromycin selection. Values represent the averages of three independent transfections, with error bars indicating standard deviations from the means.
diminish LANA’s ability to overcome RB1-mediated growth arrest.

**Alanine substitution of LANA residues 1068LKK1070 or 1125SHP1127 does not reduce LANA mediated long-term episome persistence.** We next investigated the importance of C-terminal LANA chromosome binding in establishment of episome persistence. If C-terminal LANA chromosome binding is required for episome tethering to host cell chromosomes, mutations impairing C-terminal LANA chromosome attachment should result in episome loss during cell proliferation. In initial experiments, BJAB cells alone or stably expressing similar levels of F-LANA, F-LANA1068LKK1070, or F-LANA1077FGG1079 were transfected with a plasmid containing eight copies of the KSHV TR element and conferring G418 resistance (p8TR). Transfected cells were seeded in microtiter plates at 1,000 cells/well and placed under G418 selection. After 60 days of selection, there was no difference in the amount of episomal DNA detected for F-LANA or either of the C-terminal LANA mutants as determined by Gardella gel analyses (data not shown).

To more sensitively examine the role of C-terminal LANA chromosome binding in establishment of episome persistence, we developed a limiting dilution episome maintenance assay. BJAB cells or BJAB cells stably expressing similar amounts of F-LANA, F-LANA14TG15, F-LANA1068LKK1070, F-LANA1077FGG1079, or F-LANA1125SHP1127 were cotransfected with GFP and p8TR, seeded in 96-well microtiter plates at 1, 10, and 100 cells per well, and grown under G418 selection. F-LANA14TG15 contains alanine substitutions at the indicated residues that partially disrupt N-terminal LANA binding to histones H2A/H2B and therefore to chromosomes; this mutant exhibits a reduced ability to maintain episomes (4, 5). GFP expression was monitored as a control for transfection efficiency.

In this assay, persistence of p8TR DNA, which encodes G418 resistance, permits efficient G418-resistant cell outgrowth. F-LANA-expressing BJAB cells transfected with p8TR grew out in nearly all 96 wells when seeded at 100 cells/well, in an average of 68 wells when seeded at 10 cells/well, and in 15 wells when seeded at 1 cell per well (Fig. 5A). The efficient persistence of p8TR DNA in these cells is due to LANA’s ability to mediate episome persistence. In contrast, after transfection of BJAB cells, an average of 18 wells had G418-resistant outgrowth when seeded at 100 cells/well, and 2 wells and 0 wells had outgrowth when seeded at 10 cells and 1 cell per well, respectively. BJAB cells lack LANA, and thus integration, a rare event, must occur for p8TR DNA to persist. For F-LANA14TG15, G418-resistant outgrowth occurred in an average of 63 wells at 100 cells/well, 11 wells at 10 cells/well, and 2 wells at 1 cell per well (Fig. 5A). The outgrowth of F-LANA14TG15 was less efficient than for LANA-expressing cells but more efficient than in BJAB cells. These findings are consistent with our previous observation that LANA14TG15 is reduced in its ability to mediate episome persistence (4). G418-resistant outgrowth for F-LANA1068LKK1070, F-LANA1077FGG1079, and F-LANA1125SHP1127 was similar to that of LANA. Each of these had outgrowth in nearly all wells at 100 cells/well, ~45 to 55 wells at 10 cells/well, and ~9 wells at 1 cell/well. Therefore, alanine substitutions for 1068LKK1070 and 1125SHP1127 does not reduce LANA mediated long-term episome persistence.

We next investigated the importance of C-terminal LANA chromosome binding in establishment of episome persistence, a rare event, must occur for p8TR DNA to persist. BJAB cells lack LANA, and thus integration, a rare event, must occur for p8TR DNA to persist. For F-LANA14TG15, G418-resistant outgrowth occurred in an average of 63 wells at 100 cells/well, 11 wells at 10 cells/well, and 2 wells at 1 cell per well (Fig. 5A). The outgrowth of F-LANA14TG15 was less efficient than for LANA-expressing cells but more efficient than in BJAB cells. These findings are consistent with our previous observation that LANA14TG15 is reduced in its ability to mediate episome persistence (4). G418-resistant outgrowth for F-LANA1068LKK1070, F-LANA1077FGG1079, and F-LANA1125SHP1127 was similar to that of LANA. Each of these had outgrowth in nearly all wells at 100 cells/well, ~45 to 55 wells at 10 cells/well, and ~9 wells at 1 cell/well. Therefore, alanine substitutions for 1068LKK1070 and 1125SHP1127 does not reduce LANA mediated long-term episome persistence.

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all but the F-LANA14TG15 cells, as shown by Gardella analysis. These findings are consistent with the presence of episomes in KSHV episomes but is diffusely distributed in their absence, nucleus (Fig. 5C). Since LANA concentrates to dots at sites of where F-LANA, 14TG15 diffusely distributed throughout the nucleus (Fig. 5C). During mitosis, LANA similarly concentrates to dots (Fig. 5D). These data indicate that diminished C-terminal LANA chromosome attachment does not affect localization of full-length LANA in the presence of episomes.

C-terminal LANA exerts a role on LANA chromosome attachment when N-terminal binding to chromosomes is diminished. We next investigated whether N- and C-terminal LANA might cooperate to bind chromosomes. Our initial experiments showed that mutations severely impairing C-terminal LANA chromosome binding had no effect on full-length LANA localization (Fig. 2). However, neither the 1068LKK1070 nor the 1125SHP1127 mutation completely abrogated C-terminal LANA chromosome attachment (Fig. 1) (37). It therefore remained possible that, in the context of full-length protein, N-terminal LANA binding to core histones H2A/H2B could enhance C-terminal LANA1068LKK1070 or 1125SHP1127 chromosome attachment through cooperative binding. GFP LANA was generated containing the N-terminal 14TG15 alanine substitutions in combination with alanine substitutions at either 1068LKK1070, 1077FGG1079, or 1125SHP1127. These LANA mutants were expressed in BJAB cells arrested in mitosis, and each mutant was observed for its ability to associate with chromosomes. GFP LANA 14TG15 painted chromosomes (Fig. 6, TG). Although LANA 14TG15, has reduced affinity for histones (5), we have previously observed that after transient expression it paints chromosomes nearly as well as wild-type LANA (4). Similar to GFP LANA 14TG15, GFP LANA 14TG15/1068LKK1070-8TR, and GFP LANA 14TG15/1125SHP1127-8TR broadly distributed across chromosomes (Fig. 6). In contrast, both GFP LANA 14TG15/1068LKK1070 and GFP LANA 14TG15/1125SHP1127 exhibited a lower degree of chromosome association, with most of the LANA staining occurring either between (Fig. 6) or along the periphery of chromosomes. Therefore, when N-terminal LANA chromosome binding is modestly compromised, diminished C-terminal chromosome binding reduces LANA’s association with chromosomes.

C-terminal LANA chromosome binding exerts a role in episome persistence. Since LANA’s chromosome binding is significantly reduced when both N- and C-terminal chromosome associations are compromised, we investigated whether diminished C-terminal LANA chromosome binding affects episome maintenance of LANA 14TG15, which has reduced N-terminal chromosome binding. Each of the constructs pLANA 14TG15, 8TR, pLANA 14TG15/1068LKK1070/8TR, pLANA 14TG15/1077FGG1079/8TR, and pLANA 14TG15/1125SHP1127/8TR contains eight TRs and LANA with alanine substitutions at 14TG15 and 1068LKK1070, 1077FGG1079, or 1125SHP1127. Therefore, all of these LANA mutants have reduced N-terminal chromo-
FIG. 7. C-terminal LANA chromosome association is required for efficient episome persistence when N-terminal LANA binding to histones is modestly diminished. (A) Limiting dilution episome maintenance assay. BJAB cells transfected with pZ6-8TR, pLANA14TG15-8TR, pLANA14TG15/1068LKK1070-8TR, pLANA14TG15/1077FGG1079-8TR, or pLANA14TG15/1125SHP1127-8TR were seeded in microtiter plates at 100, 10, or 1 cell/well. The number of wells with G418-resistant outgrowth was recorded after 19 days of selection. Values are averages of four independent experiments, with error bars indicating standard deviations from the means. (B) At 25 days of G418 selection, Gardella analysis was performed to detect TR episomes. 1.5 × 10^6 cells were loaded per well from cells expanded from plates seeded at 1,000 cells/well. Lane 1, BCBL-1 cells; lanes 2 and 3, BJAB cells; lanes 4 to 7, BJAB cells expressing LANA14TG15 (TG); lanes 8 to 11, BJAB cells expressing LANA14TG15/1068LKK1070 (TG/LKK); lanes 12 to 15, BJAB cells expressing LANA14TG15/1077FGG1079 (TG/FGG); lanes 16 to 19, BJAB cells expressing LANA14TG15/1125SHP1127 (TG/SHP). +, KSHV episomes from BCBL-1 cells. Vertical lines and a bracket indicate episomes. This figure is representative of four independent experiments.

some binding; those with alanine substitutions at 1068LKK1070 and 1125SHP1127 also have reduced C-terminal chromosome binding, but 1077FGG1079 does not. Each of these constructs was transfected into BJAB cells, seeded into microtiter plates, and placed under G418 selection. Averaged over four different experiments, pLANA14TG15-8TR had G418-resistant outgrowth in 92 wells at 100 cells/well, in 37 wells after seeding at 10 cells/well, and in 2 wells at 1 cell/well (Fig. 7A). pLANA14TG15/1077FGG1079-8TR, which does not have compromised C-terminal LANA chromosome binding, had G418-resistant outgrowth that was very similar to that of pLANA14TG15-8TR (Fig. 7A). In contrast, pLANA14TG15/1068LKK1070-8TR and pLANA14TG15/1125SHP1127-8TR, both of which have severely compromised C-terminal LANA chromosome binding, had markedly reduced G418-resistant outgrowth. pLANA14TG15/1068LKK1070-8TR had outgrowth in an average of 22, 2, and 0 wells for seeding concentrations of 100, 10, and 1 cells/well, respectively, while pLANA14TG15/1125SHP1127-8TR had outgrowth in an average of 36, 3, and 0 wells for 100, 10, and 1 cells/well seeding concentrations, respectively. These numbers were very similar to experiments in the absence of LANA (BJAB cells transfected with pZ6-8TR) in which outgrowth occurred in an average of 18, 1, and 0 wells for 100, 10, and 1 cells/well, respectively. Minor variations in transfection efficiency, as monitored by GFP cotransfection, or in LANA expression, as assayed by Western blotting 3 days posttransfection, did not account for the observed differences in limiting dilution outgrowth. Therefore, the 1068LKK1070 and 1125SHP1127 mutations reduced LANA14TG15-mediated persistence of TR DNA, but mutation of 1077FGG1079 did not.

To detect episomes, we performed Gardella analyses on G418-resistant cells. Since LANA14TG15 lost most episomes by day 33 (Fig. 5B) (4), we assayed for episomes after 25 days. Rapidly migrating episomes were present in all lanes for LANA14TG15 (Fig. 7B, lanes 4 to 7), and larger, slower-migrating episomes were also seen (Fig. 7B, lanes 4 and 6). As expected due to the reduced episome maintenance ability of LANA14TG15 (4), the signal intensity was moderate or low in some lanes. LANA14TG5/1077FGG1079, which does not have reduced C-terminal chromosome binding, also had episomes in all four lanes (Fig. 7B, lanes 12 to 15). In contrast, LANA14TG5/1125SHP1127, which is compromised for G418-resistant outgrowth (Fig. 7A), had no episomes in three lanes (Fig. 7B, lanes 16 to 18) and only very faint episomal signal in one lane (Fig. 7B, lane 19). LANA14TG15/1068LKK1070, which is also compromised for G418-resistant outgrowth (Fig. 7A), had no episomes (Fig. 7B, lanes 8 to 11), similar to BJAB cells, which lack LANA (Fig. 7B, lanes 2 and 3). The data in Fig. 7B are representative of results derived from four different experiments. Altogether, episomes were detected for LANA14TG15 in 16/16 cell lines, for LANA14TG15/1077FGG1079 in 15/16 cell lines, for LANA14TG15/1125SHP1127 in 4/16 cell lines, and for LANA14TG15/1068LKK1070 in 0/16 cell lines. BJAB cells transfected with Z6-8TR never had episomes. Therefore, compromise of C-terminal LANA chromosome binding in LANA14TG15/1125SHP1127 and LANA14TG15/1068LKK1070 greatly reduced LANA14TG15’s episome maintenance ability, while the mutations in 1077FGG1079, which do not compromise C-terminal binding, did not reduce LANA14TG15’s episome maintenance. This finding demonstrates that when N-terminal LANA histone H2A/H2B binding is modestly diminished, impairment of C-terminal LANA chromosome binding dramatically reduces episome persistence.

**DISCUSSION**

This work investigates the role of C-terminal chromosome binding in LANA function. Severe impairment of C-terminal LANA chromosome attachment did not affect LANA’s ability to regulate transcription from cellular promoters, overcome RBL-induced growth arrest, or maintain episomes. However, when N-terminal LANA binding to histones H2A/H2B was modestly diminished, impairment of C-terminal binding reduced both LANA’s association with mitotic chromosomes and LANA-mediated episome persistence.

In the absence of episomes, stably expressed LANA broadly
distributed across mitotic chromosomes, with preferential localization occurring on many chromosomes near centromeres and telomeres (Fig. 2). This finding refines our earlier observations in which LANA broadly associated with chromosomes without clear predilection to chromosomal subregions (2, 4). The ability to discern the pericentromeric and peritelomeric distributions in fixed cells is likely dependent on chromosome morphology and a physiologic level of LANA expression as we have observed that subchromosomal LANA distribution patterns can be masked when LANA is overexpressed, as in transient assays (36). The concentration of LANA near centromeres and telomeres is strikingly similar to the distribution of C-terminal LANA on mitotic chromosomes (36, 37) (Fig. 1).

Since N-terminal LANA diffusely paints all chromosomes (4, 61), consistent with its binding to core histones H2A/H2B (5), these observations suggest that C-terminal LANA has a direct role in LANA’s localization along mitotic chromosomes.

Surprisingly, mutations that severely impair C-terminal LANA chromosome binding had no effect on localization of full-length LANA (Fig. 2). This finding suggests that LANA does not require chromosome association of the C-terminal domain to properly target to chromosomes. Alternatively, these mutations, which impair but do not abolish C-terminal LANA attachment to chromosomes, may not be sufficient to destabilize the association of full-length LANA with chromatin. For instance, if chromosome binding of N- and C-terminal LANA is cooperative, then N-terminal LANA binding to histones H2A/H2B might enhance C-terminal LANA binding to chromatin to the extent that the impaired 1068LKK1070 and 1125SHP1127 C-terminal chromosome association is rescued. Although N-terminal LANA chromatin association is required for wild-type transcriptional regulation of some promoters (79), we now show that LANA residues 1068LKK1070 and 1125SHP1127 are dispensable for modulating BAX and CDK2 transcription. The C-terminal LANA domain is sufficient for suppressing p53-mediated BAX transcription (79); this activity was not reduced when C-terminal LANA chromosome binding was severely impaired. Similarly, although N-terminal LANA binding to chromosomes is required for wild-type activation of CDK2 transcription (79), alanine substitutions for C-terminal LANA residues 1068LKK1070 and 1125SHP1127 did not reduce the CDK2 transactivation. However, as LANA has broad transcriptional effects, we cannot rule out a role for these amino acids in modulating transcription from other cellular or viral promoters.

Although the N-terminal region is the major effector in LANA chromosome binding, this work demonstrates that C-terminal LANA chromosome attachment exerts roles in full-length LANA chromosome targeting (Fig. 6) and episome persistence that can be detected when N-terminal binding is modestly reduced (Fig. 7). Abrogating N-terminal LANA binding to histones H2A/H2B disrupts chromosome attachment of full-length LANA, resulting in loss of episome replication and persistence despite the presence of an intact C-terminal LANA chromosome binding region. Further, reduction in N-terminal LANA binding to histones H2A/H2B reduces LANA chromosome attachment, LANA-mediated episome replication, and persistence (4, 5, 44, 61). In contrast, 1068LKK1070 and 1125SHP1127 mutations that markedly diminish chromosome attachment of C-terminal LANA have no effect on full-length LANA’s chromosome localization (Fig. 2), LANA-mediated DNA replication (37), or episome persistence (Fig. 5) when N-terminal chromosome binding is wild type. However, C-terminal LANA chromosome binding is critical for LANA chromosome association and episome persistence when N-terminal LANA binding to histones is modestly diminished.

Based on these results, we propose a model in which N- and C-terminal LANA cooperatively binds to chromatin to mediate LANA chromosome association and episome persistence (Fig. 8). In this model, LANA targets chromosomes by N-terminal LANA’s binding to core histones H2A/H2B and C-terminal LANA’s interacting with another chromosome-associated factor(s) (Fig. 8) that concentrates to pericentromeric and peritelomeric chromosome regions (potential binding partners discussed in reference 36). What remains unclear is why chromosome binding of C-terminal LANA cannot efficiently rescue mutations that ablate N-terminal LANA chromosome attachment. GFP LANA5GMR7 has alanine substitutions at the indicated residues that abolish N-terminal chromosome association and histone H2A/H2B binding (4, 5).

On rare occasions we have observed GFP LANA5GMR7 to distribute as paired dots in cells on two to mitotic chromosomes, similar to the chromosome distribution of the LANA C-terminal domain (data not shown). Thus, the C-terminal LANA chromosome binding domain may partially rescue chromosome binding of LANA5GMR7, but clearly not enough to have a significant impact on DNA replication or episome persistence (since LANA5GMR7 lacks these functions) (4). The development of an in vitro binding assay would aid in understanding how the chromatin binding affinity of N- and C-terminal LANA may influence its functional contributions to LANA chromosome association and episome persistence.

LANA shares significant similarities with the viral episome maintenance proteins EBNA1 and E2 of Epstein-Barr virus and papillomavirus, respectively. In addition to maintaining viral episomal DNA, LANA, EBNA1, and E2 all regulate transcription and are involved in viral DNA replication. Although these proteins lack sequence similarity, the C-terminal DNA binding domains of E2 and EBNA1 are predicted to share significant structural similarity to the C-terminal DNA binding domain of LANA (7, 31, 37). Notably, both EBNA1 and E2, similar to LANA, contain N-terminal chromosome

![Diagram of LANA and its interactions with chromosomes](https://via.placeholder.com/150)
binding regions (4, 6, 34, 36, 40, 52, 61, 72, 81). For bovine papillomavirus type 1, E2 is targeted to mitotic chromosomes by binding to cellular Brd4 (8, 54, 84); however, this mechanism is not strictly conserved across the papillomavirus family (53, 58). Intriguingly, the E2 protein of human papillomavirus type 8 associates with mitotic chromosomes in a paired dot pattern reminiscent of that of the C-terminal LANA domain (36, 53, 58, 62), suggesting that these two proteins may share common features of chromosome association. EBNA1 has chromosome binding regions that are located between amino acids 8 to 67, 72 to 84, and 328 to 365 (34, 52, 81). Interestingly, residues 1 to 89 and residues 323 to 386 have reduced chromosome targeting abilities compared to the combination of residues 1 to 89 and residues 323 to 386. For bovine papillomavirus type 1, E2 is targeted to mitotic chromosomes by binding to cellular Brd4 (8, 54, 84); however, this mechanism is not strictly conserved across the papillomavirus family (8, 54, 84). Kaposi’s sarcoma-associated herpesvirus-like (KSHV) DNA molecules in mammalian cells by gel electrophoresis. J. Virol. 75:2882–27892.


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